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Single-Walled Carbon Nanotubes Induce Fibrogenic Effect by Disturbing Mitochondrial Oxidative Stress and Activating NF-κB Signaling

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Abstract

Single-walled carbon nanotubes (SWCNTs) are newly discovered material of crystalline carbon that forms single-carbon layer cylinders with nanometer diameters and varying lengths. Although SWCNTs are potentially suitable for a range of novel applications, their extremely small size, fiber-like shape, large surface area, and unique surface chemistry raise potential hazard to humans, including lung toxicity and fibrosis. The molecular mechanisms by which SWCNTs cause lung damage remain elusive. Here we show that SWCNTs dose and time-dependently caused toxicity in cultured human bronchial epithelial (BEAS-2B), alveolar epithelial (A549), and lung fibroblast (WI38) cells. At molecular levels, SWCNTs induced significant mitochondrial depolarization and ROS production at subtoxic doses. SWCNTs stimulated the secretion of proinflammatory cytokines and chemokines TNFα, IL-1β, IL-6, IL-10 and MCP1 from macrophages (Raw 264.7), which was attributed to the activation of the canonical signaling pathway of NF-κB by SWCNT. Finally, SWCNTs stimulated profibrogenic growth factors TGFβ1 production and fibroblast-to-myofibroblast-transformation. These results indicate that SWCNTs has a potential to induce human lung damage and fibrosis by damaging mitochondria, generating ROS, and stimulating production of proinflammatory and profibrogenic cytokines and growth factors.

Keywords

Single-walled carbon nanotubes; Human bronchial epithelial; Alveolar epithelial; Canonical signaling

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Introduction

Single-walled carbon nanotubes (SWCNTs) are molecular-scale tubes of graphitic carbon with unique electrical, chemical, and physical properties [1]. These cylindrical carbon molecules are valuable for nanotechnology, electronics, optics and other fields of materials science and technology. Conversely, the extremely small size (nano scale in diameter) and fiber-like shape of SWCNTs makes them easily became airborne and inhaled into the human lung. The high length-to-width ratio and large surface area may lead to toxic effect similar to those of asbestos fibers (mesothelioma) and silica (interstitial fibrosis) [2,3]. Evaluating the safety of SWCNTs and other nano materials in humans would help avoid the potential harms of exposing SWCNTs to workers and general population [4].

Human toxicity data concerning SWCNTs are scarce, whereas animal data are limited, and in some cases, contradictory, partly due to the fact that many parameters of SWCNTs, such as structure, size distribution, surface area, surface chemistry, charge, agglomeration state, and purity considerably affect the reactivity of carbon nanotubes with the human body. Nevertheless, current data indicated that carbon nanotubes (CNTs) including SWCNTs can enter human cells causing cell death, penetrate tissue structures to migrate and cause lesions in remote area, and induce fibrotic reactions resulting in fibrosis or mesothelioma in the lungs [5-11]. The molecular mechanism involved in the toxicities of SWCNT has not been addressed.

Although many fibrogenic particles and fibers, such as asbestos fibers and silica, cause profound fibrotic responses in the lungs of humans and animals, these materials do not appear to damage lung cells directly. Instead, they may stimulate lung epithelial, fibroblast, and macrophage cells to produce various substances that in turn induce lung lesions and fibrotic reactions. One potential culprit is reactive oxygen species (ROS). Particles and fibers may stimulate the production of ROS via three mechanisms: (a) activated macrophages and neutrophiles produce large amounts of ROS and other radicals from their respiratory burst during phagocytosis; (b) the agents damage the mitochondria to increase ROS production-mitochondria are the major organelle for oxygen consumption and ROS production under normal and many pathological conditions in mammalian cells; and (c) the surface chemistry of many particles and fibers promote ROS production [12]. Whether SWCNTs induce ROS production as a mechanism of fibrosis is currently uncertain.

NFκB is a family of transcription factors that plays critical roles in inflammation, immune response, apoptosis, and cell proliferation [13,14]. A large number of diverse external stimuli, such as infection, UV light, ROS, and cytokines lead to activation of NF-κB [15]. In unstimulated cells, NF-κB is bound with its inhibitory protein (IκB) and retained in the cytoplasm. Upon stimulation, IκB was phosphorylated and degradated by proteasomes, and thereby release NF-κB from its inhibitor protein, results in the nuclei translocation of NF-κB and where it binds to specific sequences in the promoter regions of target genes. The activation of NF-κB therefore leads to a coordinated increase in the expression of many genes whose products mediate inflammatory, immune, and fibrotic responses. Among the proinflammatory and profibrogenic cytokines and growth factors induced through the NF-

κB pathway during inflammation and fibrosis are TNFα, IL-1 β, IL-6, MCP1, and TGF β1. Whether SWCNTs activate the NF-κB pathway to influence the toxicity of SWCNTs is unclear at the present.

Mild or appropriate inflammatory response will help tissue repairing, while extensive and persistent activation of inflammatory response in pulmonary will induce aberrant matrix production in interstitial and cause lung fibrosis [16]. Lung fibrosis is a progressive, irreversible and usually fatal end stage condition of various lung diseases. Many factors associate with lung fibrosis. TGFβ1 has been considered as a critic potent regulator of extra cellular matrix formation and tissue remodeling [17]. Patient with idiopathic pulmonary fibrosis and some animal models of pulmonary fibrosis have shown increased lung TGFβ1 production [18]. Bleomycin, an anticancer drug and potent fibrogenic agent in humans, induces lung fribroblast cell differentiated into myofibroblast cells, characterized by α-smooth muscle actin (α-SMA) expression, is associated with increased expression of TGFβ1 [19]. The potential effect of SWCNTs on TGFβ1 expression and a myofibroblast function has not been addressed.

This study was conducted to identify toxicities of SWCNTs in vitro and their molecular mechanism of action. We tested the ROS generation and mitochondrial membrane potential in the contribution of SWCNT-induced cell toxicity, and investigated cytokine release, activation of the NF-κB pathway, and the fibrogenic factor TGFβ1 in the process of lung fibrosis. The purpose of the study are 1) evaluate the toxicity of SWCNTs and identify the molecular mechanism of action of SWCNTs; and 2) establish a simple and quick molecular mechanism based toxicity assessment method which can be used for high throughput screening for inflammatory/fibrogenic toxicants in vitro.

Materials and Methods

Materials

SWCNTs (2nm×2μm, purity>90%) was purchased from Sigma-Aldrich (Milwaukee, WI). A patented process that involves the decomposition of carbon monoxide over a Co-MgO catalyst at about 600°C produces SWCNTs. The material is then treated in diluted hydrochloric acid to remove of the catalyst and other metals.

Dispersion of SWCNTs

Two mg of SWCNTs were dispersed into 1 ml of the cell culture medium supplemented with 1% fetal bovine serum (FBS). The solution was mixed by vortexing for 3 times, 2 min each, followed by sonication in a tissue culture hood using a sterile sonicating probe for 3 times, 30 sec each. The 2 mg/ml stock solution was then diluted with the culture medium to appropriate concentrations and was sonicated as described above just before the experiment.

High Resolution Transmission Electron Microscopy (HRTEM)

SWCNTs were dissolved into ethanol with sonication. The samples were deposited on a 200 mesh copper TEM grid with lacy carbon film. The grid was examined using the JEOL 2100

F STEM at an accelerating voltage of 200 keV. Images were obtained at various magnifications in a bright field TEM mode.

Cell culture

BEAS-2B, a human normal bronchial epithelial cell line transformed with SV40, was cultured with a BMEM medium along with all the additives from Lonza/Clonetics Corporation (Walkersville, MD). A549, a human lung carcinoma epithelial cell line, was cultured with the DMEM medium with 10% FBS. WI38-VA13, a human normal lung fibroblast cell line, was cultured in the αMEM medium with 10% FBS. RAW 264.7, a mouse leukemic monocyte-macrophage cell line, was grown as a monolayer in the Dulbecco's modified Eagle's medium with 10% FBS. All above cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA).

Endotoxin detection

SWCNTs were prepared as suspension in water at a concentration of 2 mg/ml. The suspension was rocked on a platform for 24 h at room temperature. Samples were centrifuged for 10 min and supernatants were collected and filtered to remove remaining particles. Detection of endotoxin was performed by using the Toxin Sensor Chromogenic limulus amebocyte lysate assay kit according to the manufacturer's instructions (GenScript, Piscataway, NJ).

Cytotoxicity assay (MTT based assay)

The CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) was used to examine cell proliferation/ cytotoxicity. About 1×10^5 cells were seeded in 96-well plates for overnight. Cells were treated with SWCNTs by replacing the medium with 100 μl of the fresh medium containing varying concentrations of SWCNTs for 1, 2, and 4 days. Twenty μl of the Cell Titer 96 AQueuos One Solution Reagent was added to each well. The plates were incubated for 3 h at 37°C in a humidified incubator with 5% CO . Absorbance at 490 nm was recorded by using a 96-well plate reader. Parallel wells with no cells but having the medium containing SWCNTs at the same concentration as treated wells were used as the reference control for SWCNTs. Mean absorbance was calculated from three replicates of each exposure and subtracted from blank controls.

Reporter assay

Raw 264.7 cells stably transfected with a NF-κB binding site /Luc reporter construct was described previously [20]. Cells were treated with SWCNTs for 16 h as indicated in the figure legend. The cells were lysed with the passive reporter lysis buffer (Promega, Madison, WI) and cell lysates were vortexed and centrifuged briefly to remove cell debris. 20 μl of supernatant were mixed with 100 μl of the luciferase reagent (Promega). Luciferase activities were detected by using TD 20/20 luminometer (Agilent Technologies, Santa Clara, CA) and were normalized with protein concentrations.

Immunoblotting

Cells were lysed on ice with the RIPA buffer containing protease and phosphatase inhibitors for 30 min. Cell lysate was sonicated briefly and was centrifuged at 14,000×g for 20 min to remove cell debris. Cell lysate (10-20 μg) was fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyviinglidene difluoride (PVDF) membranes (Bio-Rad), and blocked with 5% nonfat milk in PBST (PBS plus 0.05% Tween-20). The membrane was blotted with primary antibody at 4°C for overnight with shaking, followed by incubation with horseradish peroxidase-conjugated second antibodies for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence detection reagents from Amersham Biosciences (Pisctaway, NJ). Actin was blotted as loading control.

Detection of cytokines

Mouse macrophage RAW 264.7 cells were treated with SWCNTs for 16 h or LPS (1 μg/ml) for 5 h. Cell culture supernatant was collected and stored at −80°C until use. Cytokines and chemokines were detected by using a mouse cytometric bead array kit and Flex beads (BD biosciences, San Jose, CA) and flow cytometry according to manufacturer's instruction.

ROS detection

Cells were cultured in an 8-well chamber slide and treated with SWCNTs for 16 h. Thirty minutes prior to the end of treatment; dihydroethium (hydroethidine or DHE, Invitrogen, CA) was added at 5 μM as a fluorescent indicator of ROS. Cells were washed with ice cold PBS for three times to remove free dye. Cells were then fixed with 4% paraformaldehyde and mounted with mounting solution containing DAPI for counterstaining the nucleus. Images were collected with Zeiss LSM510 confocal microscope using Rhodamin-DAPI setting. Micrographs were taken with fixed exposure times. Fluorescence intensity was quantitated using the Optimus Version 6.51 softer ware (Media Cybernetics, Silver Springs, MD). Quantitative data represent means and SDs from five separate fields for each treatment.

Mitochondrial membrane potential

Tetramethylrodamine ester (TMRE, Sigma) and Mitotrack deep red 633 (Invitrogen) are fluorescent probes that specifically accumulate in the mitochondrial matrix in a mitochondrial-inner-membrane-potential-dependent manner. Cells were cultured in an 8-well chamber slide (for confocal microscopy) or 6-well plate (for flow cytometry) and were treated with SWCNTs for 16 h. Forty-five minutes prior to the end of each treatment, 50 nM TMRE and 1 μM Mitotrack deep red 633 were added to the culture medium. Cells were washed three times with ice cold PBS. For confocal microscopy, cells were fixed with 4% paraformaldehyde in the chamber slides and were mounted with mounting solution containing DAPI to counterstaining the nucleus. Images were taken with a Zeiss LSM510 confocal microscope. For flow cytometry, cells were collected to analyze the fluorescence intensity immediately at 585nm (FL-2) and 670 nm (FL-3) channels of a FACSCalibur.

Immunofluorescent staining

Cells grown in an 8-well chamber slide were treated with SWCNTs for 16 h. Cells were fixed with 4% paraformaldehyde and permibilized with 0.5% triton X-100. Cells were stained with anti NF-κBp65 antibody (Santa Cruz Biotechnology Inc. Santa Cruz, CA) at a 1:1000 in the culture medium containing 1% FBS for 2 h. The cells were washed three times with PBS. An Alexia 488-conjugated second antibody was diluted at a 1:500 dilution in the culture medium with 1% FBS and incubated with cells for 1 h avoiding light. After washing with PBS for three times, the cells were mounted with mounting solution containing DAPI. Images were taken with a Zeiss LSM510 confocal microscope. For detection of fibroblast-to-myofibroblast transformation, RAW264.7 macrophages were treated with SWCNTs for 16 h or LPS for 5 h. The cell culture condition medium was collected and centrifuged to remove remaining cells, debris, and SWCNTs. The cell and SWCNT-free medium was designed as SWCNT-conditioned medium. Human lung fibroblast WI38-VA13 cells grown in 8-well chamber slides were cultured with the SWCNT-conditioned medium for 24 h. The cells were then fixed, permeated, and stained with anti-αSMA antibody (Sigma-Aldrich) for overnight at 4°C, followed by Alexia-488 conjugated second antibody for 1 h at room temperature. The nucleus was counterstained with DAPI. Fluorescent images were taken with a Zeiss LSM510 confocal microscope.

Reveres transcription PCR

Cells were treated with SWCNTs at 10 μg/ml for 24. IL-1β (Sino Biological Inc) at 5 ng/ ml for 24 h was used as positive control. Total RNA was isolated using Qiagen RNA mini kit. Five μg of total RNA was reverse transcripted into cDNA using reverse transcriptase III (Invitrogen). TGFβ1 cDNA was then amplified with gene specific primers (primer sequences are available upon request). GAPDH was used as an internal control.

ELISA for NF-κB DNA binding

Cells were treated with SWCNTs for 20 μg/ml for 16 h or LPS at 1μg/ml for 5 h. Nuclear extracts were prepared by using the Nuclei EZ PREP reagents from Sigma. 10 μg of nuclear lysates were added to an ELISA plate pre-coated with NF-κB - binding-site dsDNA. Bound NF-κB proteins were detected with an anti-NF-κBp 65 antibody following the instructions from Cayman (Ann Arbor, MI).

Results

Characterization of SWCNTs

The SWCNTs used in this study have a purity of large than 90% on carbon basis by X-ray diffraction. Independent laboratory tests further verified the quality of this material. High-resolution transmission electron microscopy (HRTEM) revealed the distinctive crystalline structure of a bundle of SWCNTs with each tube diameter around 2 nm and purity large than 90% (Figure 1). Elemental analyses indicated that the material contains trace amounts (μg/mg sample) of calcium 13.9, cobalt 4.3, chromium 4.8, magnesium 1.9, zinc 1.47, molybdenum 0.65, and manganese 0.19, with total metal contaminants of about 4.5%.

Endotoxin contamination of the SWCNTs was determined to be below the level of detection (maximum sensitivity of assay was 0.005 EU/ml).

To disperse SWCNTs into solution, 2 mg of SWCNTs was suspended in PBS, a dispersal medium (DM) [21], or a culture medium with 1% FBS, respectively. Agglomeration status of SWCNTs was analyzed by TEM. Large agglomerates of SWCNTs were found in the PBS suspension, whereas SWCNTs suspended in the DM or the 1% FBS culture medium were well dispersed (data was not shown). Since the culture medium with 1% FBS is compatible with the cell culture conditions, SWCNTs dispersed in a culture medium with 1% FBS were used for all the following experiments.

Cytotoxicity, ROS production, and mitochondrial damage

Bronchial epithelial, alveolar epithelial and interstitial fibroblast cells are first lines of cells to encounter inhaled stimuli. We first characterized the overall toxicity of SWCNT in human normal bronchial epithelial BEAS-2B cells, human lung alveolar epithelial A549 cells, and human lung fibroblast WI-38-VA13 cells. A modified MTT assay was used to measure cell death and proliferation. Parallel controls using the culture medium containing the same concentrations of SWCNTs as the treatment groups but no cells were used as reference to correct for light absorption from SWCNTs. As shown in Figure 2A-C, the three human lung cell lines exhibited varying, dose and time-dependant responses to SWCNTs. BEAS-2B is the most sensitive: with the 2 μg/ml SWCNTs treatment, 82% of the cells survived after 1 day of treatment and ~80% survived after 4 days; at 200 μg/ml, only 12.5% cells survived after 1 day and no cells survived after 4 days; the 20 μg/ ml treatment caused toxicities in the middle range between 2 and 200 μg/ml (Figure 2A). A549 cells were resistant to SWCNTs: after 4 days, cells survival was more than 90, 80, and 60% for 2, 20, and 200 μg/ml treatments, respectively (Figure 2B). The toxicity of SWCNTs in WI38-VA13 cells was less severe than in BEAS-2B similarly to that in A549 cells (Figure 2C).

Inducing cellular oxidative stress is a major cause of cell toxicity by fibers and particles, even though many of them do not appear to generate ROS in vitro [22,23]. We examined whether ROS production is a potential mechanism of cell toxicity by SWCNTs. Fluorescent staining experiments using a ROS-sensitive probe DHE revealed that exposure to SWCNTs at 20 μg/ml for 16 h induced production of ROS in all of the three cell lines, among which more ROS was generated in BEAS-2B cells than in A549 and WI38-VA13 cells (Figure 2D).

The mitochondria are a principal site for generation of ATP as well as ROS. Disturbing mitochondrial ROS has been link with many pathology lesions and chemical toxicities. Therefore, we examined mitochondrial damage as a potential mechanism of SWCNT-induced ROS production. TMRE and mitotrack deep red are fluorescent probes that specifically accumulate in the mitochondrial matrix in a mitochondrial-inner-membrane-potential-dependant manner. Using confocal microscopy, we found that both probes accumulated in the mitochondria in vehicle-treated cells, but accumulation was largely diminished by treatment with SWCNTs (Figure 3A). Quantitative data from flow cytometry confirmed a significant reduction of TMRE or mitotrack deep red fluorescence in SWCNT-treated cells (Figures 3B and 3C). These results indicate that SWCNTs caused substantial

loss of the electric potential across the inner membrane of the mitochondria resulting in diffusion of the probes in the cells. Together, the findings suggest that SWCNTs damage the mitochondria to increase ROS production and thereby, cause toxicity in lung cells.

Induction of proinflammatory cytokines and chemokines through NF-κB signaling

Production of proinflammatory cytokines and chemokines from macrophage and lung epithelial cells is an important step of pulmonary response to environmental stimuli. To test whether SWCNTs induce cytokine production, RAW 264.7, a widely used mouse mononuclear/ macrophage cell line, was treated with 2 or 20 μg/ml SWCNTs for 16 h. A panel of representative cytokines and chemokines were detected by using the cytometric beads array and Flex beads. TNFα, a multifunctional cytokine involved in systemic inflammation, was increased in the culture medium of SWCNT-treated cells dose-dependently (Figure 4A). IL-1β is a pro-inflammatory cytokine and was significantly increased at 2 and 20 μg/ml treatments (Figure 4B). IL-6, a proinflammatory cytokine that stimulates immune response to damaged tissues was significantly increased at 2 and 20 μg/ml (Figure 4C). MCP1, a monocyte chemoattractant of the CC chemokine family, was induced at both 2 and 20 μg/ml (Figure 4D). IL-10 is an anti-inflammatory cytokine that blocks NF-κB signaling and inhibits the production of proinflammatory cytokines, was induced strongly by LPS and SWCNTs (Figure 4E). IFN-γ, a cytokine critical for innate and adaptive immunity against viruses, intracellular bacteria, and tumor cells, was not significantly affected by LPS or SWCNTs (Data not show). IL-12, an interleukin that is naturally produced by dendritic cells in response to antigenic stimulation, was not significantly changed by either LPS or SWCNT treatment (data not show). Therefore, SWCNTs directly induce proinflammatory cytokines and chemokines including TNFα, IL-1β, IL-6, IL-10, and MCP1 to mediate inflammatory response.

NF-κB is a pivotal factor in the control of proinflammatory cytokine expression and can be activated by many stimuli. We examined whether SWCNTs activate the NF-κB pathway as a molecular mechanism for induction of the cytokines. Macrophages stably transfected with a synthetic 5x NF-κB binding site/luc reporter plasmid construct were treated with SWCNTs for 16 h. SWCNTs induced strongly the expression of the reporter luciferase (Figure 5A). This result indicates that SWCNTs could activate NF-κB promoter for gene transcription. In the canonical pathway of NF-κB signaling, NF-κB is retained in the cytoplasm by its inhibitor protein IκB in unstimulated cells and is translocated into the nucleus upon stimulation. We found that SWCNTs induced a sharp reduction in the level of IκB protein at 30 min (Figure 5B). Immunofluorescent confocal microscope results indicated that SWCNT treatment for 16 h induced a strong green fluorescence staining of NF-κB in the nucleus colocalized with DAPI stained nucleus (Figure 5C). Using a consensus NF-κB DNA-binding sequence ELISA assay, SWCNTs at 20 μg/ml for 16 h were shown to induce NF-κB binding to DNA (Figure 5D). Taken together, these results revealed that SWCNTs activate the canonical NF-κB signaling pathway to mediate the induction of proinflammatory cytokines and chemokines in macrophages.

Induction of TGFβ1 and fibroblast-to-myofibroblast transformation

A common response to many inhaled particles like silica and asbestos is the fibrogenic reaction that leads to fibrosis or mesothelioma or mesothelioma. We examined whether SWCNTs have a fibrogenic effect on lung cells. It has been reported that profibrogenic growth factors, such as transforming growth factor (TGF) β1, plays an important role in fibroblast differentiation, type I collagen deposition, and lung fibrosis. We found that the mRNA and protein levels of TGFβ1 were significantly increased in BEAS-2B (Figures 6A and 6B) and macrophage (data not shown) cells treated with 20 μg/ml SWCNTs for 24 h; the increases were comparable to those treated with IL-1β, a known inducer of TGFβ1 [24].

The de novo synthesis of α-smooth muscle actin (αSMA) by lung myofibroblasts is a hallmark of fibroblast-to-myofibroblast transformation that can be induced by profibrogenic factors released from activated macrophages or epithelial cells through a paracrine mechanism. Incorporation of αSMA into contractile fibers increases the contractive force of myofibroblasts to enhance matrix remodeling and scar formation. We analyzed the expression of αSMA in lung fibroblast cells under the treatment of SWCNTs. We first treated macrophages with SWCNTs (20 μg/ml for 16 h) or LPS (1 μg/ml for 5 h, positive control). The culture medium free of cells and SWCNTs was collected and designed as SWCNT-conditioned medium. WI 38VA-13 cells were then cultured in the SWCNT-conditioned medium for 24 h. Expression of αSMA was measured by using fluorescent microscopy with a specific antibody against αSMA. As shown in Figures 6C and D, a significant increase in the fluorescence intensity of αSMA was observed in fibroblasts cultured in the SWCNT-conditioned medium, indicating that SWCNTs stimulated macrophages to secret soluble profibrogenic factors, such as TGFβ1, which in turn function as paracrines to promote differentiation of the fibroblasts into myofibroblasts.

Discussion

Pulmonary exposure to fibrogenic agents causes damage to the lungs that eventually progresses into lung fibrosis or mesothelioma [25-28]. Mechanistic studies implicated that oxidative stress, inflammation, and production of growth factors are three major factors contributing in lung injury/repair and development of lung fibrosis [29,30]. SWCNTs are a newly discovered form of crystalline carbon, which forms cylinders of carbon having a diameter in the nanometer scale and a variable length [31,32]. Recent research indicated that carbon nanotubes such as SWCNTs could penetrate through lung tissues causing damage to the lungs and inducing lung fibrogenic lesions [8,33,34]. We use cell based molecular model to address whether SWCNTs has a fibrogenic effect and the underlining molecular events involved in the process.

ROS are produced as by-products of cellular respiration in the mitochondria or through damaging mitochondria by microorganism infection or chemical insults [12]. ROS are also formed by environmental particles via radicals on the surface or surface bound chemicals such as quinines that can undergo redox cycling to generate superoxide anions and hydrogen peroxide [29]. Last but not the least; activated macrophages and neutrophiles can produce ROS through respiratory burst [35]. Excessive ROS results in oxidative stress that promotes cell death and activates specific signaling pathways, both have being implicated in the

pathogenesis of lung fibrosis [12]. We found here that SWCNTs induced significant ROS production in bronchial epithelial, alveolar epithelial, fibroblast, and microphage cells. ROS production correlated with SWCNT-induced cell toxicity. Parallel to ROS production, SWCNTs treatment for 16 h could induce depolarization of the mitochondrial inner membrane. Our previous published result of MWCNTs [36] shown the similar toxicity pattern as SWCNTs, while the toxicity of SWCNTs are appeared earlier and more severe than the MWCNTs. Adding antioxidant, such as NAC and GSH counteract SWCNTs induced ROS in cultured cells [37,38]. Fenoglio et al. [22] used a cell-free system to show that CNTs do not directly generate free radicals. Together, these findings suggest that mitochondrial damage is likely a common mechanism by which SWCNTs induce oxidative stress and cause toxicity to lung cells.

Inhalation of CNTs induces pulmonary inflammation and fibrosis dose dependently in animals [6,39-41]. In one study, pulmonary fibrosis was observed at 7 days and granulomatous inflammation persistent throughout 56-day post-exposure period [42]. Pro inflammatory cytokines were induced in mice treated with MWCNTs [43,44]. Some studies indicated that SWCNTs induced quick interstitial fibrosis with only a transient inflammation [41]. We showed here that SWCNTs induced a panel of proinflammatory cytokines/chemokines including TNFα, IL-1β, IL-6, IL-10, and MCP1, which are commonly elevated during lung inflammation and fibrosis. We demonstrated that induction of the inflammatory mediators by SWCNTs is mediated through the canonical pathway of NF-κB signaling, as shown by the evidence that SWCNTs (1) stimulated the NF-κB response element controlled transcription of reporter gene; (2) reduced the IκB protein level in the cytoplasm; (3) increased the nuclear accumulation of the NF-κBp 65 protein; and (4) promoted binding of NF-κB to NF-κB-binding sequence in DNA.

CNTs appear to potently induce lung interstitial fibrosis and mesothelioma in rodent models [45]. Myofibroblasts are the primary collagen-producing cells characterized by expression of αSMA and collagen 1 [46]. Pulmonary mesenchymal (fibroblasts and epithelial cells) can transform into myofibroblast under certain pathological conditions. Many factors, such as growth factors TGFβ1 and PDGF, and proinflammatory cytokines TNFα and IL-1β, promote the fibroblast-to-myofibroblast transformation and subsequent matrix synthesis by myofibroblasts [19,47]. Our study using in vitro cell based model indicates that SWCNTs up-regulated TGFβ1 expression in lung fibroblast, epithelial cells, and macrophages. Furthermore, we observed that SWCNT-induced growth factors stimulate the differentiation of fibroblast into myofibrobast cells, as evidenced by the de novo synthesis of αSMA in the cells. These results demonstrate that SWCNTs is a lung inflammation and fibrogenicity inducers, which could cause lung damage and fibrosis.

Because of the extreme paucity of human data and very limited animal models for CNT safety evaluation, and of the potential high demand of the new material for the new economy worldwide, the need for rapid and efficient evaluation of CNT toxicity is apparent. Our molecular toxicity pathway based in vitro study avoided using large amount of animals, and provides a simple and quick molecular mechanism based toxicity assessment method, which can be used for high throughput screening of inflammatory/fibrogenic toxicants, such as SWCNTs, in vitro, in the future.

Acknowledgement

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

Abbreviations

CNT	Carbon Nanotubes
DHE	Dihydroethium
HRTEM	High Resolution Transmission Electron Microscopy
LPS	Lipopolysaccharide
MWCNTs	Multi-Walled Carbon Nanotubes
ROS	Reactive Oxygen Species
SMA	α Smooth Muscle Actin
SWCNTs	Single-Walled Carbon Nanotubes
TEM	Transmission Electron Microscopy
TGF	Transforming Growth Factor
TMRE	Tetramethylrodamine Ester

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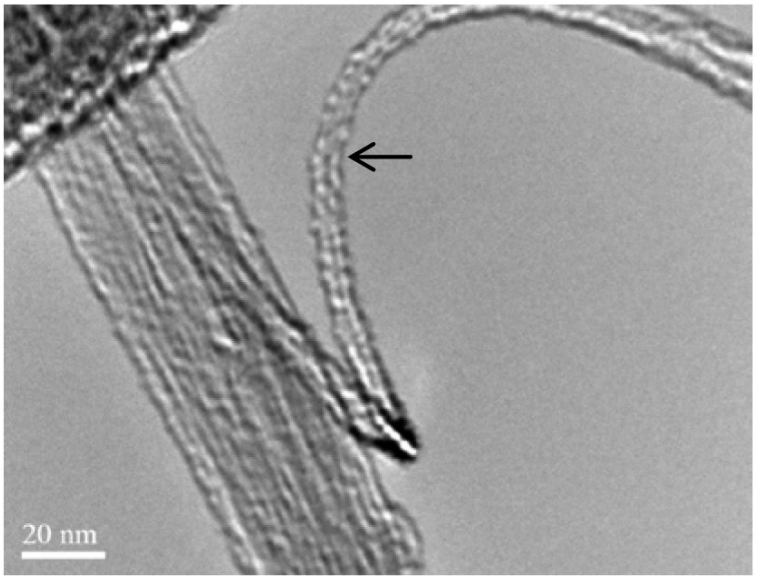

Figure 1. High-resolution transmission electron micrograph of SWCNTs

A representative crystalline structure of a bundle of SWCNT from HRTEM. Arrow shows the distinctive single-walled nanotubes structure (two tubes bound together with each diameter about 2 nm).

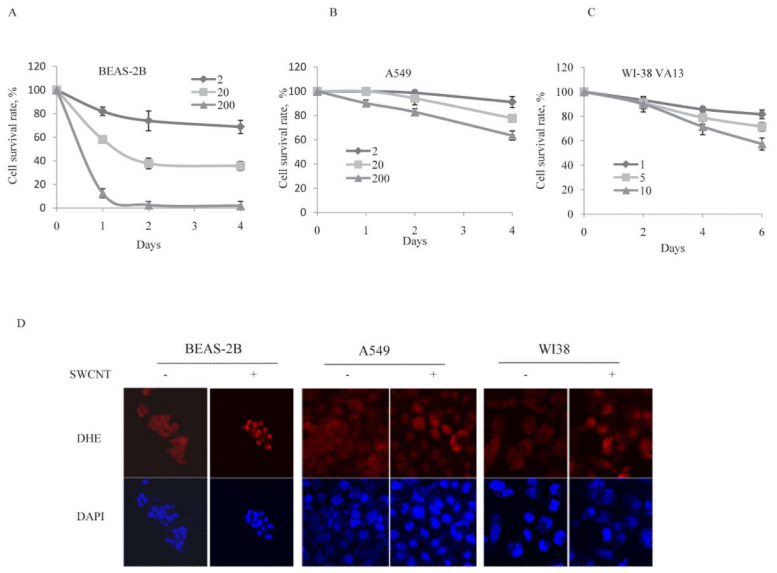

Figure 2. Cytotoxicity and ROS production in human lung cells

Cytotoxicity, BEAS-2B, WI-38-VA13, and A549 cells were cultured in 96-well plates and treated with SWCNTs from 0 to 200 μg/ml for 1 to 6 days. Cell proliferation/cell death was detected with CellTiter 96 Aqueous One Solution Cell Proliferation Assay regents. Data represent means ± SD from three samples. D. ROS production. A549, BEAS-2B, WI38-VA13 cells were cultured in 8-well chamber slides and treated with 20 μg/ml SWCNTs for 16 h. The ROS detection fluorescence probe DHE was added 30 min prior to the end of treatment. The nucleus was stained with DAPI. Image was taken using a confocal fluorescence microscope.

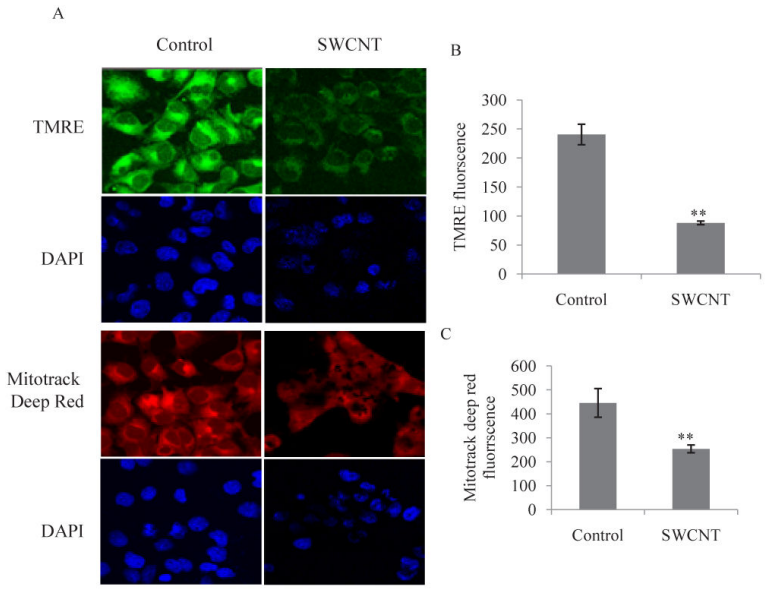

Figure 3. Effect on mitochondrial inner membrane potential

Cells were treated with SWCNTs for 16 h. Mitochondrial potential damage was detected with TMRE or Mitotrack deep red 633 using confocal microscopy (A) or flow cytometry (B & C). Data represent means ± SD from three samples. ** $p<0.01$.

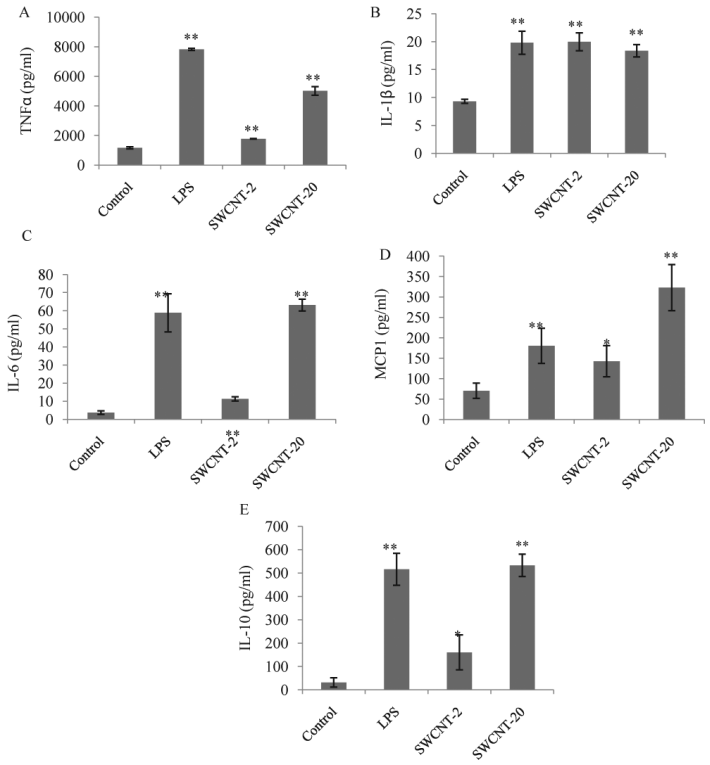

Figure 4. Induction of cytokines and chemokines

Macrophage RAW 264.7 cells were treated with SWCNTs at 0, 2 and 20 μg/ml for 16 h. LPS at 1 μg/ml for 5 h was used as a positive control. The cell-free culture medium was collected and cytokines and chemokines were detected by flow cytometry using mouse cytometric bead array and Flex beads. Data represent mean ± SD. * $p < 0.05$, ** $p < 0.01$.

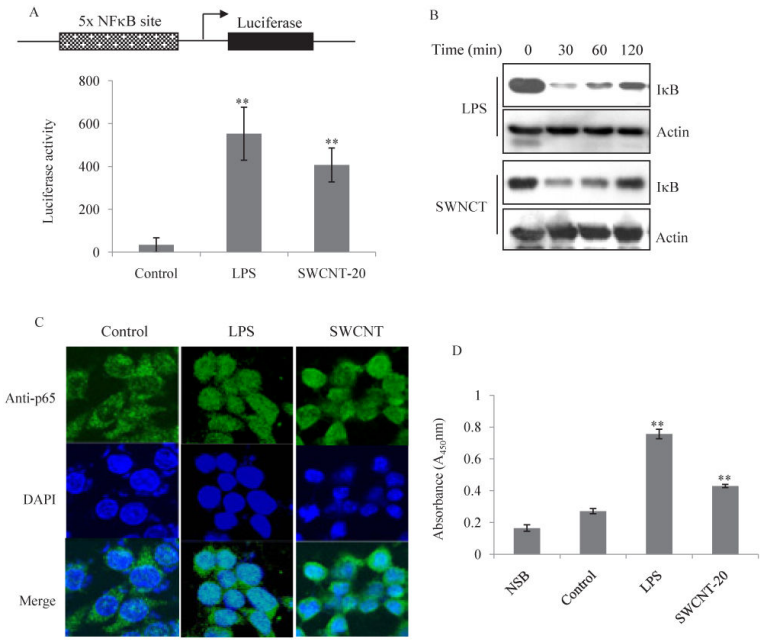

Figure 5. Activation of NF-κB signaling

A. NF-κB-binding site/Luc reporter expression. Macrophage stably transfected with 5×NF-κB –binding site/ luciferase reporter were treated with SWCNTs for 16 h. Luciferase activity from cell lysates was measured. Data represent means ± SD from three samples. **, $p<0.01$. B. IκB degradation. Macrophage cells were treated with SWCNTs at 20 μg/ml for 30, 60, and 120 min. LPS (1 μg/ml) was used positive control. Cell lysate was immunoblotted with an anti-IκB antibody. Actin was used as a loading control. C. Fluorescent microscopy of nuclear NF-κB. Cells cultured in 8-well chamber slides were treated with LPS at 1 μg/ml for 5 h or with SWCNT at 20 μg/ml for 16 h. Cells were stained with an anti-NF-κBp65 antibody, followed by Alexia 488-conjugated second antibody. Image was taken under a confocal microscope. D. Binding of NF-κB to DNA. Cells were treated with LPS at 1 μg/ml for 5 h or SWCNTs at 20 μg/ml for 16 h. Binding of NF-κB to NF-κB-binding element was measured using nuclear extracts and the NF-κB p65 ELISA assay kit. NSB, non-specific binding **, $p<0.01$.

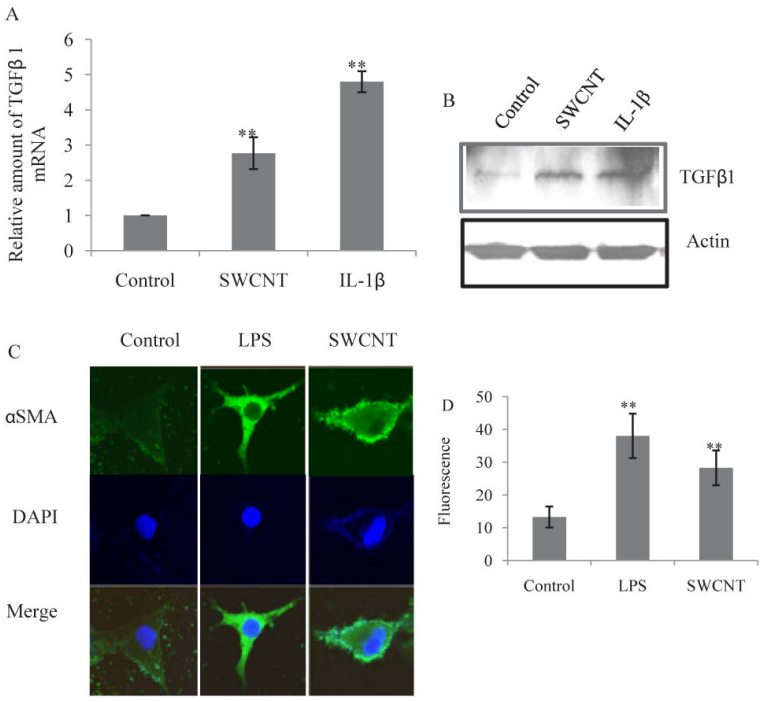

Figure 6.

Induction of fibrotic reaction. A & B Induction of TGFβ1 expression. BEAS-2B cells were treated with SWCNTs at 20 μg/ml or IL-1β at 5 ng/ml for 24 h. Real-time PCR for TGFβ mRNA (A) or immunoblotting for TGFβ protein (B) was performed. (C) Paracrine induction of αSMA. Macrophages were treated with SWCNTs (20 μg/ml, 16 h) or LPS (1 μg/ml, 5 h). The culture medium free of cells and SWCNTs were collected as the SWCNT-conditioned medium. WI38-VA13 cells were then cultured in the SWCNT-conditioned medium in 8-well chamber slides for 24 h. Expression of αSMA was detected by immunofluorescent staining of the cells with an anti-αSMA antibody followed by an alexia-488 conjugated second antibody. The nucleus was stained with DAPI. Cell images were taken under a Zeiss LSM510 confocal microscope. (D) Quantitative data of C from 5 separate fields.